Interleukin-6 and Tumor Necrosis Factor α -Mediated Expression of Hepatocyte Growth Factor by Stromal Cells and its Involvement in the Growth of Endometriosis

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Abstract

Background: The regulation of hepatocyte growth factor (HGF) by basal and lipopolysaccharide-stimulated macrophages in women with endometriosis has been recently demonstrated. However, as a cell component of endometriosis, the expression of HGF by stromal cells and its regulation by other inflammatory mediators is limited. Therefore, we investigated the expression of HGF gene and protein by the stromal cells derived from women with or without endometriosis and its regulation by interleukin-6 (IL-6) and tumor necrosis factor α (TNF α). **Methods:** Using primary culture, stromal cells as immunoreactive to vimentin were isolated from the eutopic and ectopic endometrium of 18 infertile women with endometriosis and 12 women without endometriosis. The production of HGF in the culture media of basal and IL-6 or TNF α -stimulated stromal cells was examined by ELISA. The mRNA expressions of HGF and its receptor, c-Met in the stroma were investigated by reverse transcription-polymerase chain reaction (RT-PCR). The localization of HGF and c-Met in isolated stromal cell and in intact tissue was examined by immunohistochemistry. The effect of HGF on the growth of stromal cells either alone or in combination with IL-6 or TNF α was examined by

bromodeoxyuridine (BrdU) incorporation study. **Results:** The production of HGF in the culture media of stromal cells was significantly increased after single or combined treatment of either IL-6 or TNF α when compared with non-treated cells. IL-6 or TNF α mediated production of HGF was inhibited by anti-IL-6, anti-TNF α or anti-HGF antibody. The production of HGF by the stromal cells derived from the eutopic endometrium of women with endometriosis was significantly higher than that from non-endometriosis. This effect of IL-6 or TNF α in increasing HGF at the protein level was in parallel with the increased expression of HGF and c-Met mRNA as demonstrated by RT-PCR. HGF and c-Met were also immunolocalized in isolated cells and in intact tissue. BrdU incorporation study indicated that the addition of HGF enhanced the growth of endometrial and endometriotic stroma either alone or in combination with IL-6 or TNF α and this effect was partly inhibited after addition of anti-HGF antibody. **Conclusion:** IL-6 and TNF α are involved in the production of HGF by endometrial stromal cells and may be involved in the growth of endometriosis in an autocrine mechanism.

Key Words: cell growth / endometriosis / hepatocyte growth factor / IL-6/ TNF α .

Introduction

Endometriosis is characterized by the presence and growth of endometrial-like tissue (gland and stroma) outside the uterus and occurs in 10% of reproductive age women (Straphy et al., 1982). The pathogenesis of endometriosis is still debatable. A line of evidence has already demonstrated that an innate immune system and ovarian steroid hormones participate either alone or in an orchestrated fashion in the regulation of this disease (Khan et al., 2005a; Akoum et al., 2000).

Recent study (Khan et al., 2005b) suggests that activated macrophages are increased in the peritoneal fluid of women with endometriosis. These activated macrophages secrete numerous macromolecules that may contribute to the implantation of endometrial cells and the progression of endometriosis (Buyalos et al., 1992; Donnes et al., 1998; Eisermann et al., 1988). There is no doubt that besides ovarian steroid hormones, the regulation in the growth or maintenance of endometrial or endometriotic tissues is contributed by a number of cytokines and growth factors such as interleukin (IL)-6, IL-8, tumor necrosis factor-α (TNFα), vascular endothelial cell growth factor (VEGF) and hepatocyte growth factor (HGF) (Fujishita et al., 1999; Khan et al., 2003, 2005a, 2005b; Harada et al., 2001). Among these different cytokines and growth factors,

HGF has been reported to possess divergent biological activities in the invasion, growth or metaplastic transformation of endometrial tissue and pelvic mesothelium (Ishimaru et al., 2004; Khan et al., 2003; Yoshida et al., 2004).

A line of literature has already demonstrated that hepatocyte growth factor (HGF) is elevated in the PF of women with endometriosis than healthy controls (Khan et al., 2002, 2004a; Osuga et al., 1999). Besides the paracrine mode of action, the activity of HGF in an autocrine fashion has already been demonstrated in isolated endometrial stroma, stroma of endometriotic cyst and macrophages derived from women with endometriosis (Khan et al., 2005a; Yoshida et al., 2004).

We have recently demonstrated (Khan et al., 2005a) that HGF can be regulated in endometrial cells and inflammatory cells by basal and lipopolysaccharide-stimulated macrophages and is involved in the growth of endometriosis. Since endometriosis is a regurgitated product of uterine endometrium, pelvic endometriotic tissues are bathed in the peritoneal fluid enriched with different cytokines. In addition, HGF promoter region retains the responsive element for IL-6 and TNF α (Zarnegar R, 1995), so it is possible that the production of HGF can also be regulated by IL-6 and TNF α in endometrial

stromal cells. However, studies are limited in the regulation of HGF using stromal cells derived from either eutopic or ectopic endometrium of pelvic endometriosis. The only report by Sugawara et al. (1997) demonstrated a significantly increased secretion of HGF by basal stromal cells derived from the eutopic endometrium of women with endometriosis than that in non-endometriosis.

Therefore, we investigated the production of HGF by endometrial stromal cells in response to IL-6 and TNF α both at the protein and transcriptional levels. In addition, we examined the immunolocalization of HGF and its receptors, c-Met in isolated stroma and intact tissues. Finally, we demonstrated the growth promoting effect of exogenous HGF either alone or in combination with IL-6 or TNF α on the stromal cells derived from the eutopic and ectopic endometrium of women with or without pelvic endometriosis.

Material and Methods

Subjects. For the measurement of HGF, IL-6 and TNF α concentration in peritoneal fluid (PF), a total of 30 women between 22 and 38 years of age undergoing laparoscopy for pelvic pain, dysmenorrhea and/or infertility were recruited in this study. A total of 16 women between 23 and 39 years of age who were free of endometriosis by

laparoscopy formed the control group. Endometriosis was diagnosed laparoscopically and histologically. The extent of the disease was staged according to the revised classification of the American Society of Reproductive Medicine (r-ASRM) (1997). Neither the study group nor the endometriosis free group had been on hormonal medication in the 3months prior to the surgical procedure. The detail clinical characteristics of women with or without endometriosis are described in a separate study (Khan et al., 2002).

All women with or without endometriosis had regular menstrual cycles (28-32 days). All induced menstrual cycles were excluded from the current study. For the isolation of stromal cells in primary culture, biopsy samples were collected from either eutopic or ectopic endometrium of 18 women with endometriosis and 12 women without endometriosis. All biopsy specimen and peritoneal fluid were collected in accordance with the guidelines of the Declaration of Helsinki and with the approval by the Nagasaki University Institutional Review Board. An informed consent was obtained from all women.

Isolation and culture of stromal cells. Stroma was collected from the biopsy

specimens of the eutopic and ectopic endometrium derived from the women with or without endometriosis. The detail procedure of the isolation of stroma is described previously (Osteen et al. 1989; Sugawara et al., 1997; Khan et al. 2005a).

The characteristics of the cultured stromal cells were determined by morphological appearance and immunocytochemical studies against human vimentin monoclonal antibody (stromal cell specific) at a dilution of 1:20 (V9; Dako) and as described recently (Khan et al., 2005a). The isolated cells were placed in four-chamber slide (Nunc, Naperville, IL). After 24 hours, the slides were washed in PBS, fixed with 4% paraformaldehyde for 10 minutes, and rinsed with PBS. Slides then were incubated in 0.1% Triton X-100 for 5 minutes and incubated for 3 hour at 37℃ with the antibodies as follows to exclude the contamination with other cells: against human cytokeratin monoclonal antibodies (mAb) (epithelial-cell specific) at a dilution of 1:50 (MNF 116;Dako, Denmark), against human von Willebrand factor mAb (endothelial-cell specific) at a dilution of 1:50 (Dako), and against CD45 mAb (other leukocytes) at a 1:50 (Dako) dilution. The specificity of the immunocytochemical staining was confirmed by the deletion of the first antibody. Immunocytochemical staining was performed on at least

three different isolated cells with similar results.

HGF assay in the culture media of treated and non-treated stromal cells. The culture media of basal (non-treated) and stimulated (1, 10, and 50ng/mL each of IL-6 and TNF α) stromal cells were prospectively collected and assays were performed retrospectively in each of six women with endometriosis and non-endometriosis (three in the proliferative phase and three in the secretory phase of menstruation). The concentrations of HGF in the serum free culture media were measured in duplicate using a commercially available sandwich enzyme linked immunosorbent assay (ELISA) developed by R & D system in a blind fashion (Quantikine, R & D system, Minneapolis, MN). The antibody used in HGF determination does not cross-react with other cytokines. The use of anti-HGF antibody did not affect this assay system. The limit of detection was 40.0 pg/mL for HGF. Both the intra-assay and inter-assay coefficients of variation were <10% for all these assays. The neutralizing effect on HGF secretion in the culture media was also performed by a 4 hour pre-incubation with each of anti-IL-6 antibody, anti-TNF α antibody and anti-HGF antibody (10 μ g/mL for each) and then treated the cells with either IL-6 or TNF α (10ng/mL for each) for another 24 hour without washing the

pre-incubated antibodies.

Immunolocalization of HGF in isolated cells and in intact tissue. In order to immunolocalize HGF and its receptor, c-Met in vimentin-immunoreactive isolated stromal cells and in intact tissue, we performed immunohistochemistry using respective antibody and using paraffin-embedded tissue sections of eutopic and ectopic endometrium derived from each of six women with or without endometriosis (three in the proliferative phase and three in the secretory phase). A 1:50 dilution of a rabbit polyclonal antibody against a recombinant protein of HGF α (H-145) (sc-7949; Santa Cruz Biotechnology, Santa Cruz, CA) and a 1:20 dilution of mouse monoclonal antibody raised against NCL-c-Met receptor (clone 8F11; Novocastra Laboratories Ltd., Newcastle, UK) of human origin was used.

Immunohistochemistry was performed in the 5 μ m thick serial section of paraffin-embedded tissues as we described previously (Khan et al., 2003, 2005a). Non-immune mouse immunoglobulin (Ig) G1 antibody (1:50) was used as a negative control. Placental tissue, which is known to exhibit high levels of HGF, was used as a positive control. The immunostaining was quantified by a modified method of

quantitative-histogram score (Q-H score) as described recently (Khan et al., 2003; Ishimaru et al., 2004). The Q-H score was calculated using the following equation: Q-H score= Σ Pi (i+1), where i = 1, 2 or 3 and Pi is the percentage of stained cells for each intensity. The staining intensity was graded as 0 = no, 1 = weak, 2 = moderate, and 3 = strong. We calculated the mean Q-H scores of five different fields of one section by light microscopy at moderate magnification (x200).

Determination of HGF and c-Met mRNA by RT-RCR in Stromal Cells. We have used the RT-PCR technique to determine the mRNA levels of HGF and its receptor, c-Met in basal (non-treated) and stimulated stromal cells (treated by 1, 10, 50ng/mL each of IL-6 and TNF α either alone or in combination) as derived from women with or without endometriosis. Total ribonucleic acid (RNA) was isolated from each of 10^6 stromal cells cultured in 60 mm petridish (Greiner) using the monophasic solution of 40% phenol and ISOGEN method (Molecular Research Center, Tokyo), according to the manufacturer's protocol.

Reverse transcription of RNA extracted from cultured endometrial stromal cells into complementary DNA and PCR amplification was performed as we

described recently (Khan et al. 2005a). Human oligonucleotide primers of HGF, c-Met and β -actin as we used for our current study, their location on cDNA and corresponding GenBank accession numbers were described previously (Khan et al. 2005a).

The amplification reaction was initiated by heat denaturation at 94℃ for 1 minute, annealing of the primers for I minute at 59℃, and then extension for 1 minute at 72℃. This was repeated for 32 cycles for each of HGF and c-Met using the PCR apparatus (Takara Biomedicals, Tokyo). The amplification protocol for β -actin used as an internal control was same as above except annealing condition of the primer (62℃ for 1 minute) and the reaction was repeated for 23 cycles. After the final cycle, the temperature was maintained at 72℃ for 10 minutes to allow completion of synthesis of amplification products. Although not shown, a control with no reverse transcription was run with each sample to confirm that PCR products were free of DNA contamination.

Analysis of PCR-amplified products was performed by fractionation over a 1.5% agarose gel followed by ethidium bromide staining of DNA bands. A scanner densitometer was used to determine the ratio of intensity of each band relative to β -actin and is represented as the relative expression of the target gene. This relative expression is

defined by the fold increase of the mRNA band intensity comparing to the basal levels (0 control and normalized to 1) as we also described recently (Khan et al., 2005a). Densitometric analysis of gel bands was performed using the National Institutes of Health image analysis program.

Cell Proliferation Assay by Bromodeoxyuridine (BrdU) Incorporation Study. Proliferation of the stromal cells derived from eutopic and ectopic endometrium was determined spectrophotometrically by measuring the incorporation of BrdU into the replicated cells. The detail procedure of BrdU incorporation assay was described recently (Khan et al. 2005a). The absorbance values correlated directly to the amount of DNA synthesis and thereby to the number of proliferating cells in culture. The cell proliferation study was performed after single or combined treatment with recombinant IL-6, TNF α or HGF (R & D system).

In order to confirm that besides IL-6 and TNF α, growth-promoting factor in the treated culture medium is contributed by HGF, we used antibody to deplete HGF in the conditioned medium. Cells were pre-treated with anti-HGF antibody (10 μ g/mL, R & D system), incubated for 4 hr and then again treated with exogenous IL-6, TNF α or

HGF for another 24 hrs without washing the pre-incubated antibody and then examined the changes in cell growth.

Statistical Analysis. The clinical characteristics of the subjects were evaluated by one-way analysis of variance. The data are expressed as either mean ± SEM or mean ± SD. The distribution of each result was initially analyzed by F-test. When F-test indicated skewed distribution, we applied non-parametric statistical analysis such as Mann-Whitney U-test. When F-test indicated normal distribution of the results between groups, we applied parametric statistical analysis such as Student's t-test. Since the concentration of HGF in the culture media was not normally distributed, HGF levels between endometriosis and non-endometriosis were analyzed by non-parametric test. Other continuous variables between groups were compared with Student's t-test. Spearman's rank order correlation test was used to determine the correlation between each of two markers. A power calculation was performed to assess the number of patients required in each of proliferative and secretory phase to see a statistically significant ($p=0.05$) difference in the means of 500pg/mL of HGF at a power of 80%. Differences were considered as statistically significant for $p<0.05$.

Results

There were no significant differences in clinical characteristics between women with or without endometriosis (data not shown). We and others previously demonstrated that the levels of HGF, IL-6 and TNF α in PF were significantly higher in women with endometriosis than that in non-endometriosis (Khan et al. 2002, 2004a; Harada et al. 2001). For the current study, we further analyzed the relationship between HGF and IL-6 or between HGF and TNF α in the PF of 30 women with endometriosis. We found a significant positive correlation between the levels of HGF and IL-6 ($r=0.476$, $p=0.0079$; Figure 1A) or between HGF and TNF α ($r=0.685$, $p<0.0001$; Figure 1B). We didn't find any correlation between any of these two markers in 16 women without endometriosis (data not shown).

HGF production by single and combined treatment of IL-6 and TNF α. After an initial time-depended study, we found that HGF concentration in the culture media after IL-6 and TNF α treatment peaked at 24hr to 48hr (data not shown). There fore, in our all following studies, we cultured all treated-and non-treated cells for a period

of 24 hr. As shown in Figure 2A, HGF production in the culture media was more increased in women with endometriosis (4-6 fold) than that of non-endometriosis (3-4 fold). Although, a peak increase was noticed at 10ng/mL each of IL-6 and TNF α than that of either at 1ng/mL or 50ng/mL and also when compared with non-treated stromal cells (Figure 2A), a synergistic effect between IL-6 and TNF α (each of 10ng/mL and 50ng/mL) on increasing HGF production was equally observed in women with or without endometriosis. However, no significant difference was observed between single treatment and combined treatment of these cytokines in women with either endometriosis or non-endometriosis.

When we compared the secretion of HGF by the basal and treated stromal cells between women with endometriosis and non-endometriosis, we found that basal (non-treated) stromal cells of endometriosis secreted more HGF in the culture media than that from non-endometriosis (180.4 ± 14.2pg/mL vs. 120.1 ± 2.5pg/mL, p=0.06), although this difference did not reach a statistical significance. However, HGF production by the stromal cells of endometriosis was significantly higher in women with endometriosis than that from non-endometriosis after single treatment with 10ng/mL of

IL-6 (478.2 ± 20.6pg/mL vs. 321.5 ± 26.2pg/mL, $p<0.05$) or after combined treatment with IL-6 and TNF α (642.2 ± 55.0pg/mL vs. 461.4 ± 25.8pg/mL at 10ng/mL each, $p<0.05$; and 613.5 ± 19.9pg/mL vs. 493.2 ± 7.2pg/mL at 50ng/mL each, $p<0.05$; Figure 2A). Although an apparent increase in the secretion of HGF was observed after treatment with 10ng/mL or 50ng/mL of TNF α and combined treatment with each of 1ng/mL of IL-6 and TNF α in women with endometriosis, no significant difference was achieved when compared with similarly treated cells of non-endometriosis (Figure 2A).

We also didn't find any difference in the secretion of HGF between proliferative phase and secretory phase of menstrual cycle (data not shown). A sample size calculation indicated that 50 patients in each group would be required to detect with 80% power a significant difference ($p=0.05$) in the value of HGF between proliferative and secretory phases of menstruation.

Since culture media of IL-6 and TNF α -treated stromal cells retain other macromolecules in addition to HGF and in order to confirm that the secreted product in the culture media is HGF after stimulation and its production is mediated by IL-6 and TNF α , we extended our experiment by using antibody to deplete IL-6, TNF α or HGF in

the conditioned media of stromal cells derived from women with endometriosis. This neutralization effect of respective antibody on the production of HGF is shown in Figure 2B. We found that although not significant, the blocking effect of IL-6, TNF α or HGF tended to decrease the production of HGF in the culture media towards its production by non-treated stromal cells (Figure 2B). This indicates that besides other cytokines and growth factors, the treated culture medium contains HGF and its production is mediated by IL-6 and TNF α .

Immunolocalization of HGF and c-Met in isolated stromal cells and in intact tissue. We previously demonstrated that immunostaining of HGF and c-Met in the eutopic endometrium of women with endometriosis was more intense than in that of control women (Khan et al. 2003). Besides, immmunoreaction of HGF in the epithelium and stroma of eutopic endometrium derived from women with endometriosis was stronger than that of control women (Khan et al. 2003). In our current study, we further confirmed the tissue localization of HGF and its receptor, c-Met in isolated stromal cells (Figure 3b and 3c, respectively) and in intact tissue derived from the eutopic endometrium (Figure 3e and 3f, respectively) and ectopic endometrium (Figure 3h and 3i,

respectively) of women with endometriosis. The corresponding negative controls as shown by the immunoreaction to nonimmune mouse IgG are shown in Figure 3a, 3d and 3g. Although data not shown, this immunoreaction of HGF and c-Met in the isolated stroma and intact tissue and as measured by Q-H score was found to be stronger than that in similar cells or tissues of women without endometriosis. We did not find any significant variation in the immunoreaction of these markers between the proliferative phase and secretory phase of menstruation (data not shown). This indicates that HGF is being synthesized by stromal cells and after binding with c-Met, HGF may confer an autocrine mode of action.

mRNA expression of HGF and c-Met by IL-6 and TNF α -treated stromal cells. In order to determine if the regulation of HGF and its receptor, c-Met, also occurs at the transcriptional level, we examined HGF and c-Met mRNA expression in stromal cells derived from the eutopic endometrium of women with or without endometriosis and after single or combined treatment with IL-6 and TNF α (Figure 4).

The RT-PCR of HGF, c-Met and β -actin mRNAs gave rise to bands of 505, 536 and 300bp, respectively (Figure 4). In women with endometriosis, HGF mRNA

expression was found to be stronger in women with endometriosis than that in non-endometriosis after single treatment of either IL-6 or TNF α (Figure 4A and 4B) and this expression of HGF mRNA was more increased after combined treatment of IL-6 and TNF α (Figure 4C). However, c-Met mRNA expression was found to increase in a dose-dependent manner and this was equally observed for women with and without endometriosis (Figure 4A and 4B).

After densitometric analysis, a 2.0-to 2.5-fold increase in the expression of HGF mRNA in stromal cells was found in women with endometriosis after treatment with 10ng/mL ($p<0.05$) and 50ng/mL ($p<0.05$) each of IL-6 or TNF α (Figure 5A). A low level expression of HGF mRNA was observed in women without endometriosis at the similar treatment of IL-6 and TNF α (Figure 5A). However, these levels of HGF expression were greater than that of basal production or either of the cytokines alone in women without endometriosis.

The differences in the relative expression of HGF and c-Met mRNA between the stromal cells of women with endometriosis and without endometriosis are shown in Figure 5. Similar to the increased secretion of HGF in the culture media as shown in

Figure 2A, the mRNA expression of HGF and its receptor, c-Met was also significantly higher by the stromal cells of endometriosis than that of non-endometriosis (Figure 5A and 5B). The statistical differences between endometriosis and non-endometriosis are as follows: HGF mRNA, at 10ng/mL and 50ng/mL of IL-6 ($p<0.05$ for both) and at 50ng/mL of IL-6+TNF α , ($p<0.05$); c-Met mRNA, at 10ng/mL each of IL-6 and TNF α ($p<0.05$ for both) and at 50ng/mL of IL-6+TNF α ($p<0.05$).

The production of HGF at both gene and protein levels under the current stimulation protocol was independent of either the proliferative phase or the secretory phase of menstrual cycle (data not shown).

Effects of IL-6, TNF α and HGF on stromal cell proliferation. Since exogenous HGF stimulated a maximum proliferation of endometrial epithelial cells and stromal cells with a concentration of 10-50ng/mL as we described recently in a dose-dependent study (Khan et al. 2005a), cell proliferation study of stromal cells was carried out with 50ng/mL of recombinant HGF in the current study. Again, effect of exogenous IL-6 and TNF α on stromal cell proliferation was carried out with a concentration of 10ng/mL, because both IL-6 and TNF α stimulated a peak HGF

production in the culture media at this concentration.

We found that single or combined treatment of stromal cells with exogenous IL-6, TNF α and HGF was able to stimulate significant proliferation of stromal cells derived from the eutopic endometrium of women with endometriosis and non-endometriosis as measured by BrdU incorporation (expressed by fold increase and normalized to 0 control) (Figure 6A). We observed that after single treatment of either IL-6, TNF α or HGF, stromal cells derived from women without endometriosis were unable to significantly incorporate BrdU when compared with non-treated cells. However, stromal cells of non-endometriosis significantly proliferated after their combined treatment (IL-6 + HGF, $p<0.05$ and TNF α + HGF, $p<0.05$, Figure 6A). In contrast, stromal cells derived from women with endometriosis were equally responsive to either single or combined treatment with IL-6, TNF α and HGF (TNF α , $p<0.05$; HGF, $p<0.05$; IL-6+HGF, $p<0.05$; TNF α +HGF, $p<0.05$; Figure 6A) when compared with non-treated cells. After parametric analysis, the differences of BrdU incorporation between endometriosis and non-endometriosis are as follows: HGF, $p<0.05$; IL-6+HGF, $p<0.05$; TNF α +HGF, $p<0.05$ (Figure 6A).

Since BrdU incorporation study represents the simple incorporation of BrdU into the proliferated DNA of these cells and does not reflect the actual cell growth as accounted by increased cell number, therefore, we also tried to examine the cell growth of stroma by cell number (initial plating 10^5cells/mL/well and expressed by fold increase as normalized to 0 control)) under IL-6, TNF α and HGF stimulation (Figure 6B). We found a parallel and significantly increased cell growth under a stimulation dose of 10ng/mL of TNF α ($p<0.05$), 50ng/mL of HGF ($p<0.05$) and also by the combined effect of IL-6 and HGF ($p<0.01$) or TNF α and HGF ($p<0.01$) for women with endometriosis and under IL-6 + HGF ($p<0.05$) or TNF α + HGF ($p<0.05$) for women without endometriosis (Figure 6B). The differences in cell growth between endometriosis and non-endometriosis are as follows: basal stroma, 34.0 ± 3.4x10^5 cells/mL vs. 23.7 ± 1.9x10^5cells/mL, $p=0.08$; treated stromal cells by HGF, IL-6 + HGF, and TNF α + HGF ($p<0.05$ for all, Figure 6B).

Finally when attempted to confirm that combined proliferative effect of IL-6 and HGF or TNF α and HGF on stromal cells was partly contributed by HGF and not exclusively by IL-6 or TNF α, we used anti-HGF antibody to block the effect of

exogenous HGF in the culture media. We found that proliferation of stromal cells as induced by combined IL-6 and HGF or TNF α and HGF was partly or significantly inhibited after the depletion of HGF in the culture media. This was observed by both BrdU incorporation study (Figure 6A) and also by cell growth as measured by the number of stromal cells (Figure 6B). In fact, anti-HGF antibody significantly inhibited IL-6+HGF-mediated cell growth in women with endometriosis and non-endometriosis ($p<0.05$ for both, Figure 6B). Although not shown, anti-HGF antibody had a tendency to suppress increased BrdU incorporation and cell number by TNF α treatment in endometriosis (+) group. This indicates that the growth or persistence of endometrial and endometriotic tissues in pelvic microenvironment is an orchestrated effect of different macromolecules including HGF.

We also studied the exogenous effect of IL-6, TNF α and HGF on stromal cells derived from ectopic endometrium of women with endometriosis (Figure 7). We found that BrdU incorporation into the stromal cells of peritoneal lesions (Figure 7A) and cell growth (Figure 7B) were higher in response to different cytokines when compared to similar cells of corresponding eutopic endometrium (2.0 to 4.5 fold vs. 1.5 to 3.5 fold,

respectively). It was interesting to observe that stromal cells derived from eutopic endometrium of endometriosis was not responsive to IL-6 alone in increasing cell proliferation, however, single treatment with IL-6 was able to significantly proliferate the stromal cells of ectopic endometrium ($p<0.05$, Figure 7A and 7B). Again, depletion of HGF in the culture media after pre-treatment with anti-HGF antibody suppressed IL-6+HGF- or TNF α +HGF-mediated increase of stromal cell proliferation derived from ectopic endometrium. This further indicates that HGF may contribute to the growth of both eutopic and ectopic endometrial stromal cells.

Similar to eutopic endometrium, we also did not find any difference in cell growth of basal or treated stroma of ectopic endometrium between the phases of menstrual cycle. When we compared the difference of stromal cell proliferation (both basal and treated) between eutopic and ectopic endometrium, we found the statistical differences between them as follows: IL-6, $p<0.05$; IL-6+HGF, $p<0.05$ or TNF α +HGF, $p<0.05$ for both BrdU incorporation and cell growth (Figure 7A and 7B). It was also interesting to observe that IL-6+HGF- and TNF α +HGF-mediated cell proliferation and growth of ectopic endometrial stromal cells were more resistant to suppression than that

of eutopic endometrium after application of anti-HGF antibody (Figure 7A and 7B).

Discussion

We demonstrated that besides regulation of HGF by isolated macrophages under the stimulation of lipopolysaccharide in pelvic microenvironment (Khan et al. 2005a), HGF could also be regulated in endometrial stromal cells in response to other inflammatory mediators such as IL-6 and TNF α . We reported here that the production of HGF was significantly elevated in the condition media of stromal cells derived from women with endometriosis than that of women with non-endometriosis under the stimulation of IL-6 and TNF α either alone or in combination. This interaction between HGF and cytokines at the cellular level was in accordance with their significant correlation in the PF of women with endometriosis in pelvic environment.

We further demonstrated in our current study that the increased secretion of HGF in the media conditioned by stromal cells and its corresponding immunolocalization in isolated stromal cells and in intact tissue were in parallel with their increased transcriptional activity at the mRNA level of HGF and its receptor, c-Met under the similar stimulation. There was a decreasing tendency of HGF secretion in the culture

media after application of corresponding antibody against IL-6 and TNF α . In addition, IL-6 and TNF α -mediated stromal cell proliferation was also suppressed by anti-HGF antibody. This indicates that the production of HGF is mediated by either IL-6 or TNF α and the growth promoting effect of endometrial and endometriotic stromal cells may also be contributed by HGF itself in addition to IL-6 and TNF α .

Sugawara et al. (1997) previously reported a significantly increased secretion of HGF by non-treated eutopic endometrial stromal cells in women with endometriosis than that in women without endometriosis. We also found an increasing tendency of HGF secretion by the basal (non-treated) endometrial stromal cells derived from women with endometriosis than that from women without endometriosis. Although we expected a similar significance between them but this elevation of HGF in the culture media did not reach a statistical significance. There are some reasons in the discrepancy of HGF secretion between these two studies. First of all, we cultured stromal cells for a period of 24 hour instead of 48 hour and in a serum free medium whereas Sugawara et al., (1997) used 2.5% fetal bovine serum in their culture media. Secondly, the detection limit of HGF was 0.1ng/mL (100pg/mL) by their study and <40pg/mL by our assay system. Both the

intra-assay and inter-assay coefficients of variation were <10% for our assay. Irrespective of the difference in these two assay systems, we can at least postulate that endometrial stromal cells of endometriosis are biologically more active than that of non-endometriosis.

If we consider that pelvic endometriotic lesions are bathed in the PF, it is very logical to assume that PF enriched with different cytokines and growth factors including HGF, IL-6 and TNF α could stimulate the growth and progression of endometriosis. In fact, different studies demonstrated that PF of women with endometriosis contains increased concentrations of HGF, IL-6 and TNF α than that of women without endometriosis (Khan et al., 2002; Harada et al., 2001). The origin of HGF, IL-6 and TNF α in pelvic environment could be from endometrial cells or mesothelial cells (Song et al., 2003; Ishimaru et al., 2004). In addition, the origin of these macromolecules could be also from the infiltrated macrophages into ectopic endometrium and corresponding eutopic endometrium (Khan et al., 2004b).

HGF has a diverse biological effect ranging from cell mitosis, angiogenesis, migration, and metaplastic transformation to invasion of cells into pelvic mesothelium.

Therefore, we believe that regulation of HGF by IL-6 and TNF α in the endometrial stromal cells has some biological significance. HGF could be involved in the regeneration of endometrium after menstruation by its mitogenic activity or retrograde migration of menstrual debris by its motogenic activity. Recently Yoshida et al. (2004) demonstrated that HGF/c-Met system plays a role in the pathogenesis of endometriosis by its cell invasion activity after regurgitated endometrial cells enters into the pelvic microenvironment. In fact, we observed in our current study that HGF either alone or in combination with IL-6 or TNF α was able to more proliferate ectopic stromal cells when compared with eutopic stromal cells. This additional cell growth and cellular resistance to growth suppression by antibody against HGF in pelvic endometriotic lesions could be responsible for the persistence or progression of pelvic endometriosis.

The exact mechanism of this additional cell growth by ectopic stromal cells is unknown. However, according to some recent publications, it has been claimed that ectopic tissue differs from eutopic endometrium by its proliferation rate, steroid hormones levels and markers of apoptosis (Beliard et al., 2004; McLaren et al., 1997). Therefore, our current findings of more increased cell proliferation by ectopic sromal

cells could be explained by increased sensitivity to proliferation and their resistance to apoptosis as compared to similar cells of eutopic endometrium. This could promote the dissemination and implantation of these cells to ectopic sites. Despite this biological differences between ectopic and eutopic endometrium, we did not find any influence of the phases of menstrual cycle in the proliferation of ectopic tissues.

The autocrine and paracrine regulation of HGF between cells of mesenchymal origin and epithelial cells or within the same endometriotic stromal cells have been reported in several literatures (Khan et al., 2005a; Yoshida et al., 2004). We also recently reported mRNA expression of HGF and its receptor, c-Met in the infiltrated macrophages derived from women with endometriosis and established that there is an autocrine, paracrine or intracrine relationship among endometrial epithelial cells, stromal cells and infiltrated macrophages (Khan et al., 2005a). However, information regarding the regulation of HGF by IL-6 and TNF α in the endometrial stromal cells has been lacking.

An interaction between TNF α and IL-6 with the consequent up-regulation of either IL-6 or IL-8 gene and protein and their involvement in the proliferation of endometriotic stromal cells derived from chocolate cyst has been reported (Iwabe et al.,

2000). However, there is no study in the regulation of HGF by IL-6 and TNF α in women with pelvic endometriosis. Therefore, we demonstrated for the first time using the endometrial stromal cells derived from women with pelvic endometriosis and found that IL-6 and TNF α either alone and in combination could effectively induce the expression or production of HGF at both gene and protein levels.

In our recent study (Khan et al., 2005a), we reported that as an initial inflammatory mediator, LPS could regulate the production of HGF by macrophages and this could enhance the cell proliferation of endometrial epithelial cells, stromal cells and also infiltrated macrophages. We further demonstrated that exogenous HGF either alone or in combination with IL-6 or TNF α was able to significantly proliferate the endometrial stromal cells derived from women with pelvic endometriosis. This mitogenic activity of HGF was higher in women with endometriosis than that in women without endometriosis. Besides other cytokines and growth factors, the contribution of HGF in this enhanced proliferation of stromal cells was confirmed by decreasing tendency of stromal cell proliferation after application of anti-HGF antibody.

Finally we conclude that in addition to its production by inflammatory cells or

other mesenchymal cells, HGF may also be produced by endometrial stromal cells derived from women with pelvic endometriosis, its regulation can be mediated by IL-6 or TNF α which are the constant component of PF in pelvic microenvironment and their orchestrated effect may be involved in the growth of endometriosis.

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Figure Legends

Figure 1. Shows the correlation between the levels of HGF and IL-6 (**A**) or between HGF and TNF α (**B**) in the peritoneal fluid of 30 women with pelvic endometriosis. A significant positive correlation was observed between the PF levels of HGF and IL-6 (r=0.476, p=0.0073) or between HGF and TNF α (r=0.685, p<0001). No difference was observed between each of these two markers in 16 women without endometriosis (data not shown).

Figure 2. Shows the dose-dependent production of HGF in the culture media of isolated stromal cells under the single or combined treatment of IL-6 or TNF α derived from the eutopic endometrium of six women with pelvic endometriosis and six women without endometriosis (**A**) (three each in the proliferative phase and three in the secretory phase of menstruation). The asterisk denotes the significantly different from cells of non-endometriosis at the indicated doses IL-6 and TNF α (*p<0.05, **A**). The neutralizing effect on the IL-6 or TNF α - mediated production HGF after application of respective antibody against IL-6, TNF α or HGF demonstrated a partial inhibition of HGF production in the culture media in endometriosis (+) group (**B**). The results are expressed as mean $\pm$ SEM of duplicate determinations of six different patients. There was no

difference in HGF secretion between the phases of menstruation in either of these two groups of women.

Figure 3. Shows the immunohistochemical localization of HGF (middle panel) and its receptor, c-Met (right panel) in the vimentin-positive isolated stromal cells (***b*** and ***c***), in the intact tissue of eutopic endometrium (***e*** and ***f***) and ectopic endometrium (***h*** and ***i***) of the same women with pelvic endometriosis. The corresponding negative controls as shown by the immunoreaction to nonimmune mouse IgG are shown in Figure 3a, 3d and 3g (left panel). Besides isolated stromal cells (***b*** and ***c***), HGF and c-Met was found to be immunoreactive in the stromal cells as well as in glandular epithelial cells of both eutopic (***e*** and ***f***) and ectopic endometrium (***h*** and ***i***). Final magnification was adjusted at x50 and using light microscope connected to a camera (Olympus-VANOX, model-AHBS, Tokyo, Japan).

Figure 4. Shows the single (**A**, **B**) and combined treatment (**C**) of a variable concentrations of IL-6 and TNF α (0-50ng/mL) on mRNA expression of HGF and its receptor, c-Met in isolated stromal cells derived from the eutopic endometrium of women without endometriosis (**A**) or with pelvic endometriosis (**B, C**) and as detected by

RT-PCR. The transcript sizes of HGF and c-Met were found at the indicated gel bands. The expression of HGF and c-Met mRNA was found to be stronger in women with endometriosis (**B, C**) than that in non-endometriosis (**A**). β -actin was used as an internal control.

Figure 5. Shows the densitometric analysis of individual mRNA band of HGF and c-Met as found in Figure 4 (**A**, **B** and **C**) after single and combined treatment with IL-6 and TNF α. The band intensity was initially normalized with the corresponding band of internal control (β -actin) and was represented by the fold increase (relative expression) relative to 0 control (without treatment with IL-6 or TNF α) and normalized to 1. The asterisk (*) denotes the significantly different from non-endometriosis. For both HGF and c-Met (**A** and **B**), *$p<0.05$ vs. non-endometriosis at the indicated doses of IL-6 and TNF α. The results are expressed as mean $\pm$ SEM of three different experiments derived from three separate patients from each group.

Figure 6. Shows the bromodeoxyuridine (BrdU) incorporation (**A**) and cell growth (**B**) as measured by counting the number of cells (initial plating, 10^5cells/mL/well) after single or combined treatment with IL-6, TNF α or HGF, and after application of a neutralizing

antibody to HGF to measure the changes in stromal cell proliferation and cell growth derived from the eutopic endometrium of women with or without pelvic endometriosis. The exogenous addition of HGF significantly stimulated stromal cell proliferation and cell growth either alone or in combination with IL-6 or TNF α (**A** and **B**). The blocking effect of anti-HGF antibody reversed the cell proliferation towards non-treated cells (**A**, **B**). The results are represented as fold increase of cell proliferation and cell growth relative to 0 control (without treatment). The results are expressed as mean $\pm$ SEM of three different experiments derived from three separate patients. The asterisk (*) indicates the significantly different from non-treated cells (*$p<0.05$ vs. 0 control for **A**; *$p<0.05$, **$p<0.01$ vs. 0 control for **B**). Unlike BrdU incorporation, anti-HGF antibody significantly suppressed HGF-and IL-6-mediated cell growth (*$p<0.05$, **B**).

Figure 7. Shows the bromodeoxyuridine (BrdU) incorporation (**A**) and cell growth (**B**) after single treatment or co-treatment of HGF with either IL-6 or TNF α in the stromal cells derived from the eutopic and ectopic endometrium of women with pelvic endometriosis. The results are represented as fold increase of cell proliferation by BrdU incorporation (**A**) or cell growth by cell number (**B**) relative to 0 control (without

treatment). It was interesting to observe that BrdU incorporation and cell growth of endometriotic stromal cells in response to IL-6, TNF α or HGF was more increased when compared to similar cells of eutopic endometrium (**A** and **B**). *p<0.05 and **p<0.01 vs. 0 control for both BrdU incorporation (**A**) and cell growth (**B**). Similar to eutopic stromal cells, addition of anti-HGF antibody tended to reverse HGF+IL-6- mediated or HGF+TNF α -mediated cell growth (**A** and **B**) of ectopic stroma.

Figure 1

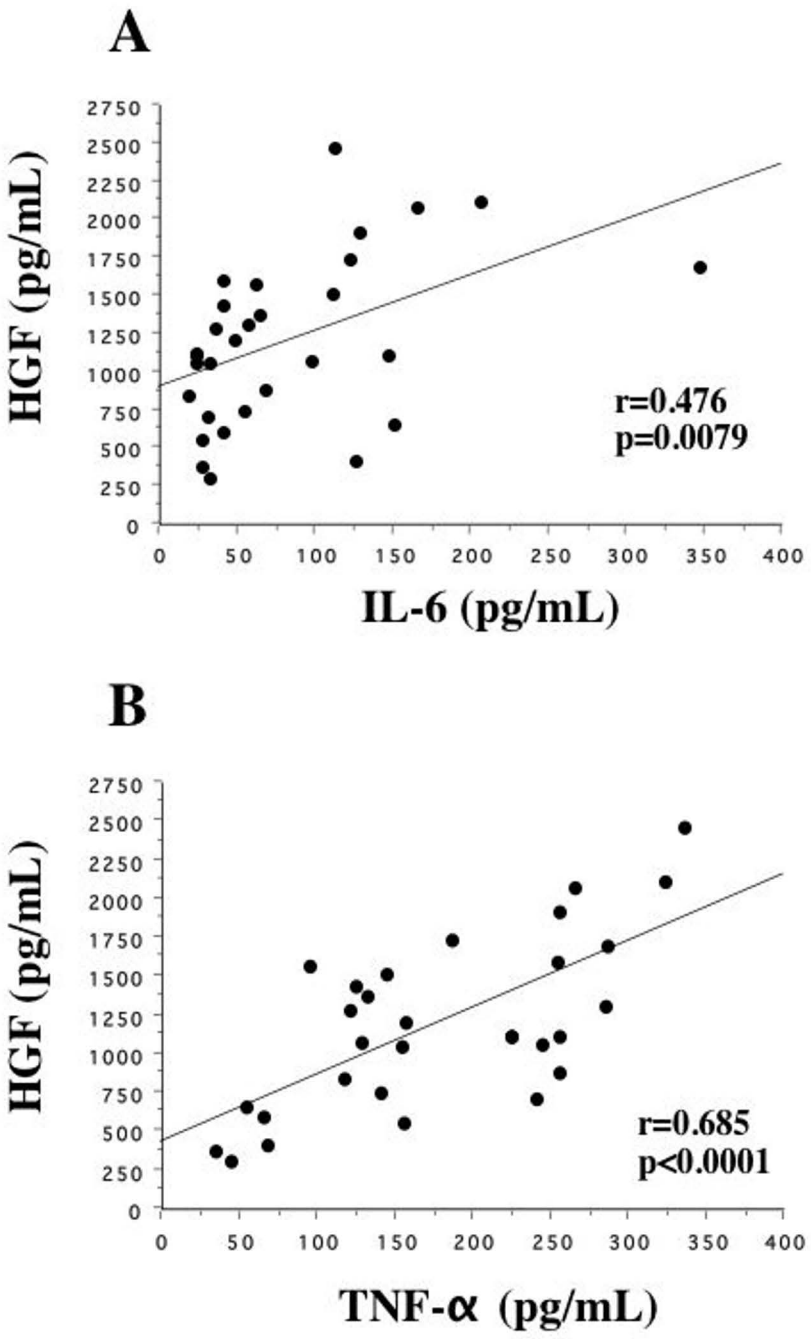

A
HGF (pg/mL)
IL-6 (pg/mL)
r=0.476
p=0.0079
B
HGF (pg/mL)
TNF-α (pg/mL)
r=0.685
p<0.0001

Figure 2

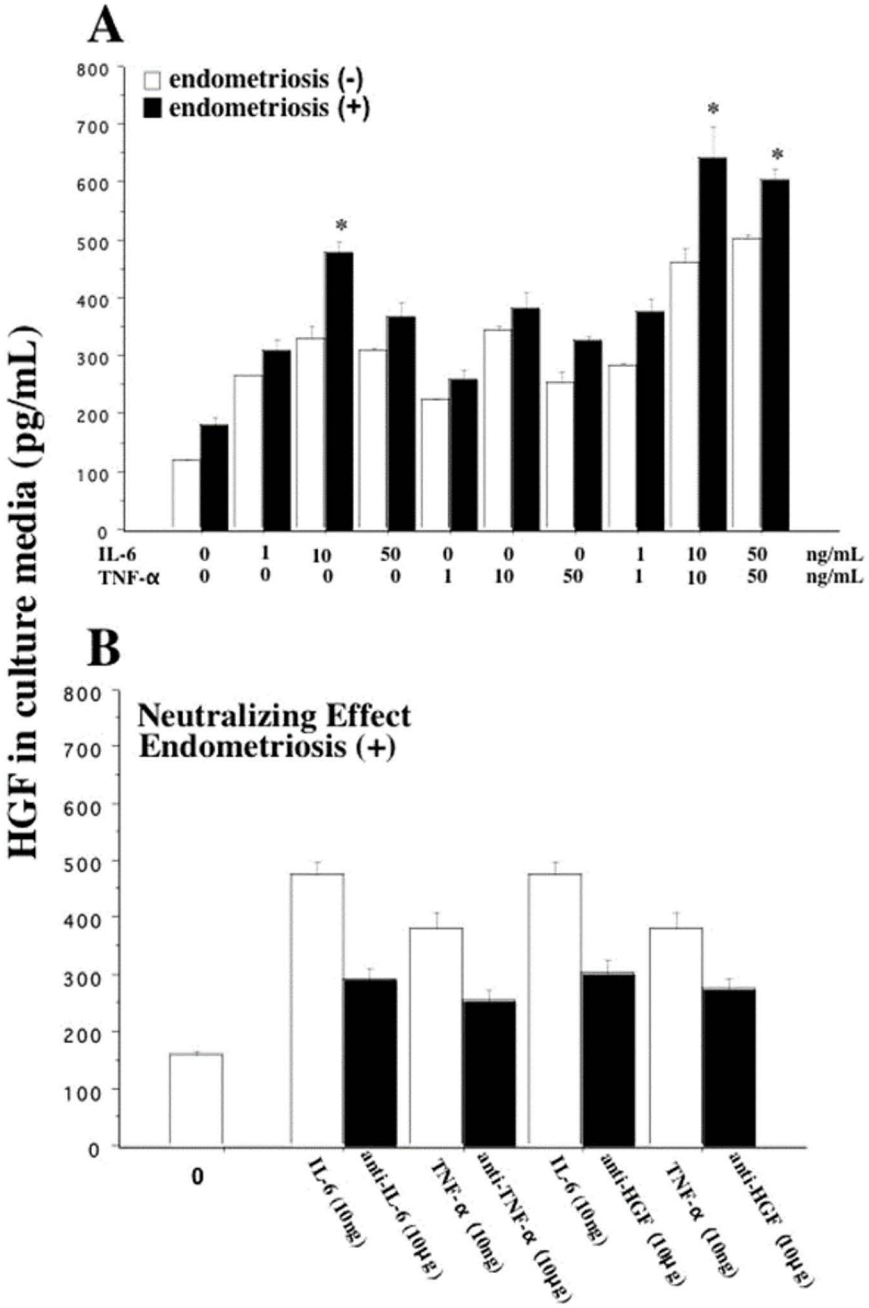

A
endometriosis (-)
endometriosis (+)
HGF in culture media (pg/mL)
IL-6 0 1 10 50 0 0 0 1 10 50 ng/mL
TNF-α 0 0 0 0 1 10 50 1 10 50 ng/mL
B
Neutralizing Effect
Endometriosis (+)
0
IL-6 (10ng)
anti-IL-6 (10μg)
TNF-α (10ng)
anti-TNF-α (10μg)
IL-6 (10ng)
anti-HGF (10μg)
TNF-α (10ng)
anti-HGF (10μg)

Figure 3

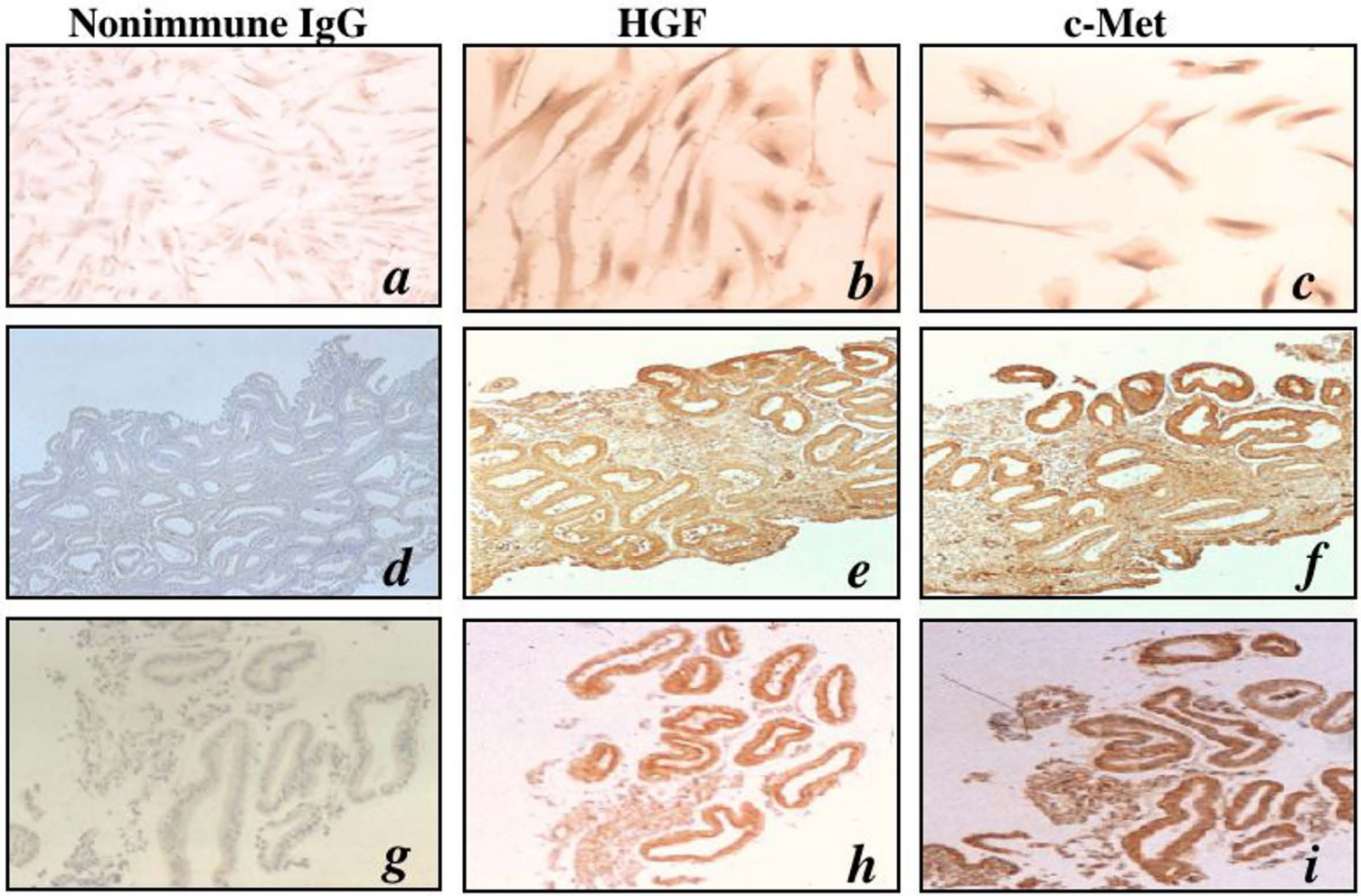


Figure 4

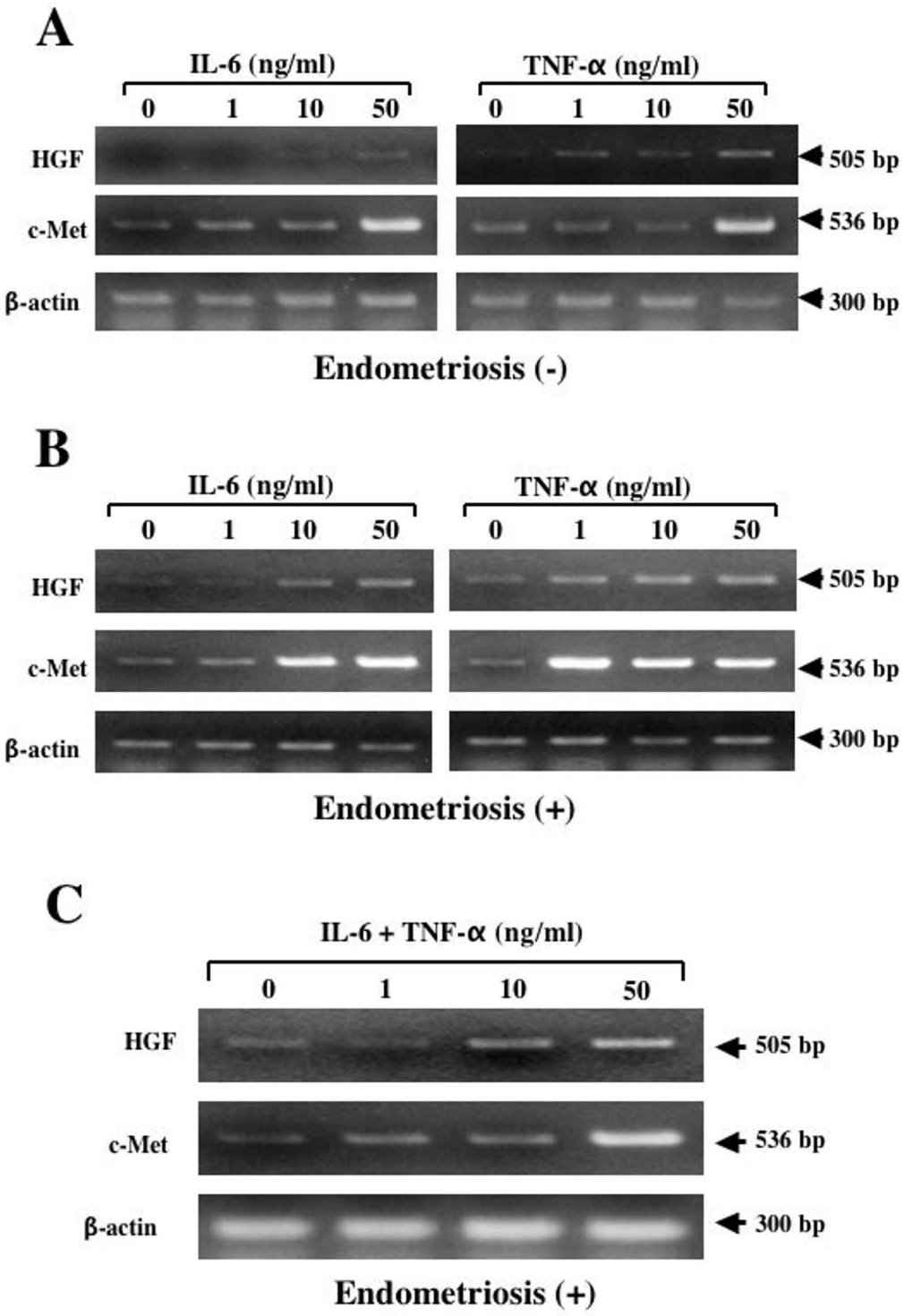

A
IL-6 (ng/ml)
TNF-α (ng/ml)
0 1 10 50
0 1 10 50
HGF
c-Met
β-actin
505 bp
536 bp
300 bp
Endometriosis (-)
B
IL-6 (ng/ml)
TNF-α (ng/ml)
0 1 10 50
0 1 10 50
HGF
c-Met
β-actin
505 bp
536 bp
300 bp
Endometriosis (+)
C
IL-6 + TNF-α (ng/ml)
0 1 10 50
HGF
c-Met
β-actin
505 bp
536 bp
300 bp
Endometriosis (+)

Figure 5

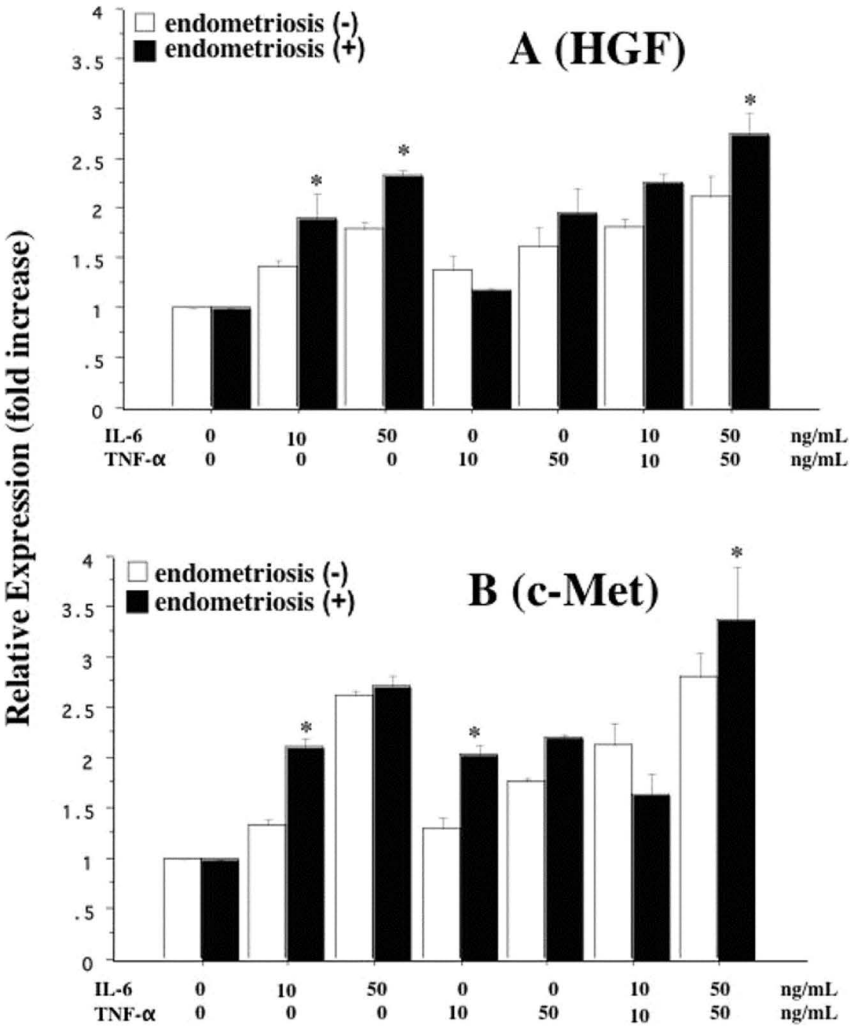

Relative Expression (fold increase)
endometriosis (-)
endometriosis (+)
A (HGF)
B (c-Met)
IL-6 0 10 50 0 0 10 50 ng/mL
TNF-α 0 0 0 10 50 10 50 ng/mL

Figure 6

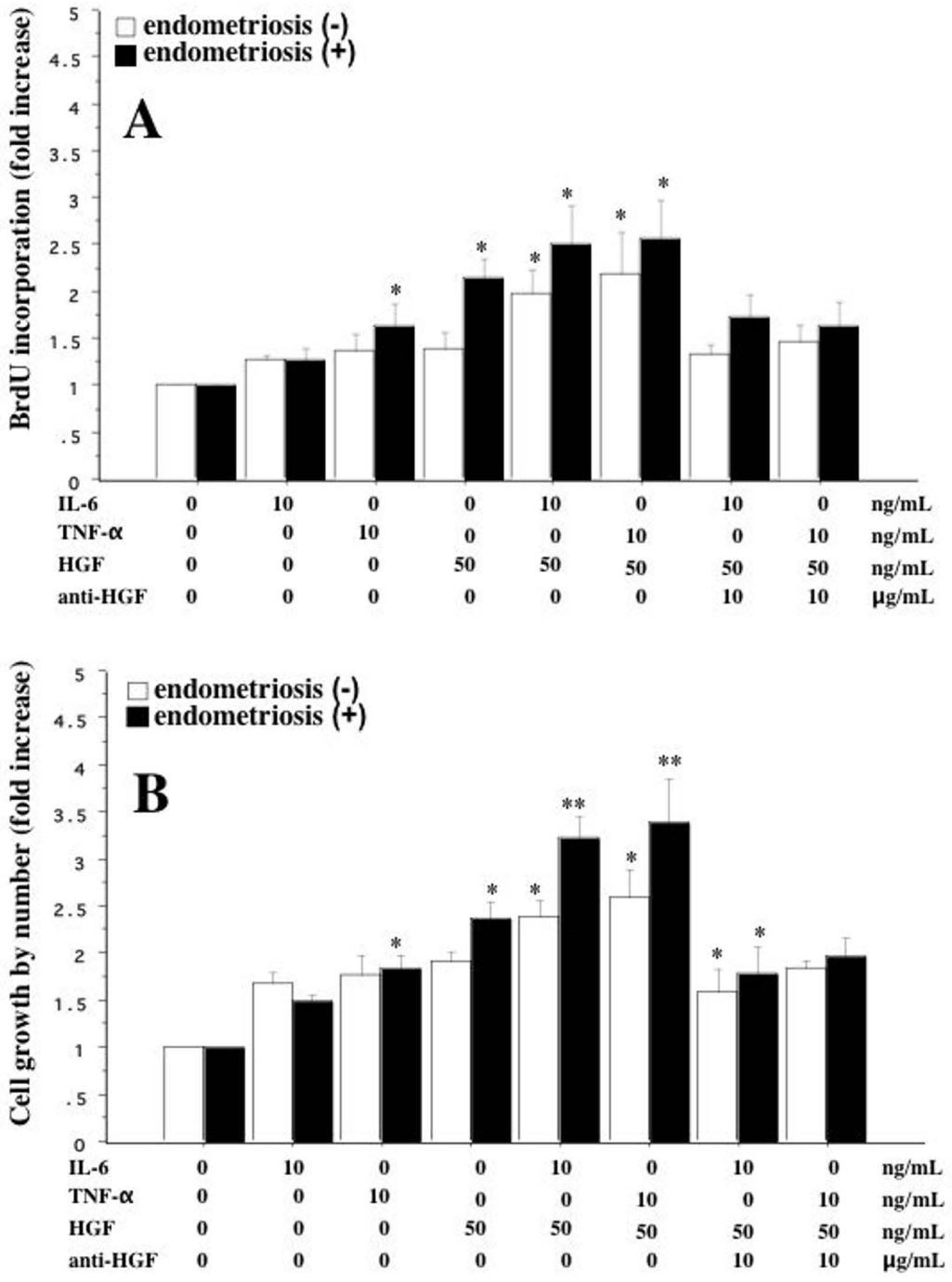
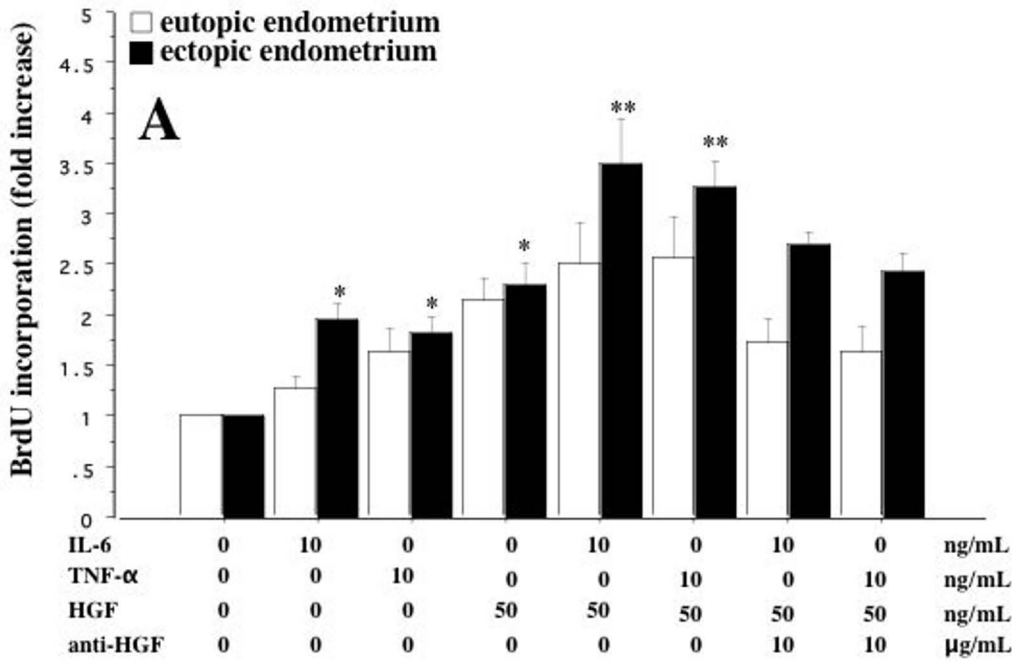
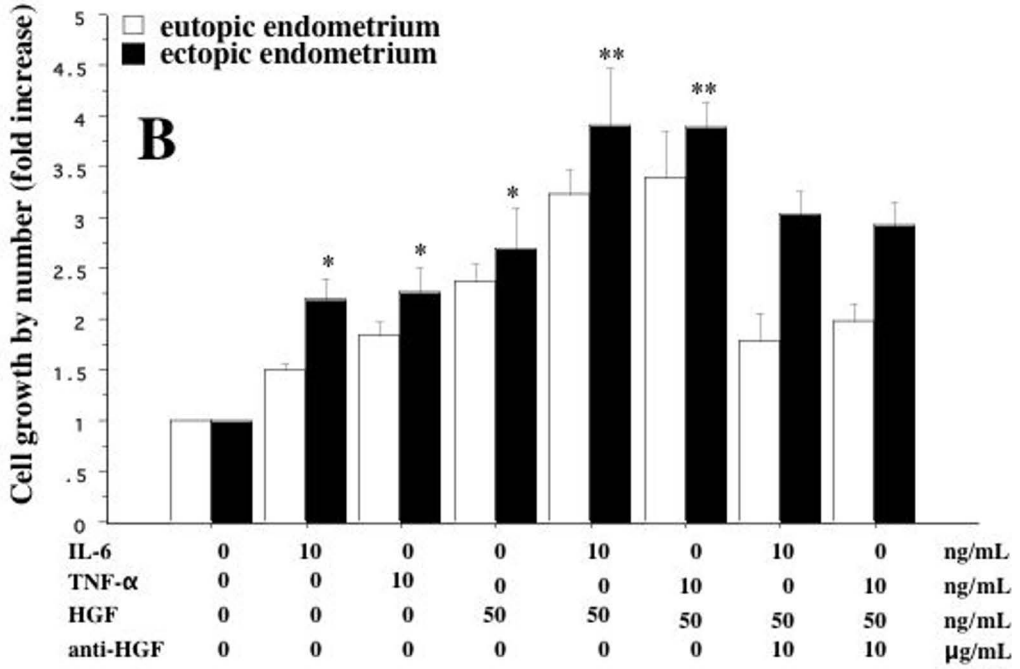

endometriosis (-)
endometriosis (+)
A
BrdU incorporation (fold increase)
5
4.5
4
3.5
3
2.5
2
1.5
1
.5
0
IL-6 0 10 0 0 10 0 10 0 ng/mL
TNF-α 0 0 10 0 0 10 0 10 ng/mL
HGF 0 0 0 50 50 50 50 50 ng/mL
anti-HGF 0 0 0 0 0 0 10 10 μg/mL
endometriosis (-)
endometriosis (+)
B
Cell growth by number (fold increase)
5
4.5
4
3.5
3
2.5
2
1.5
1
.5
0
IL-6 0 10 0 0 10 0 10 0 ng/mL
TNF-α 0 0 10 0 0 10 0 10 ng/mL
HGF 0 0 0 50 50 50 50 50 ng/mL
anti-HGF 0 0 0 0 0 0 10 10 μg/mL

Figure 7


eutopic endometrium
ectopic endometrium
A
BrdU incorporation (fold increase)
0
.5
1
1.5
2
2.5
3
3.5
4
4.5
5
*
**
IL-6 0 10 0 0 10 0 10 0 ng/mL
TNF-α 0 0 10 0 0 10 0 10 ng/mL
HGF 0 0 0 50 50 50 50 50 ng/mL
anti-HGF 0 0 0 0 0 0 10 10 μg/mL


eutopic endometrium
ectopic endometrium
B
Cell growth by number (fold increase)
0
.5
1
1.5
2
2.5
3
3.5
4
4.5
5
*
**
IL-6 0 10 0 0 10 0 10 0 ng/mL
TNF-α 0 0 10 0 0 10 0 10 ng/mL
HGF 0 0 0 50 50 50 50 50 ng/mL
anti-HGF 0 0 0 0 0 0 10 10 μg/mL